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Methanotrophic activity in slurry surface crusts as influenced by CH_4, O_2, and inorganic N

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Abbreviations

MOB: Methane oxidizing bacteria; PLFA: Phospholipid fatty acid; FAME: Fatty acid methyl esters

Keywords

Methane oxidation; Inorganic nitrogen; Microsensor; PLFA stable-isotope probing; Methane oxidizing bacteria.

Core Ideas

- Oxygen penetration into surface crusts is shallow.
- Nitrous oxide accumulates at oxic-anoxic interfaces in surface crusts.
- Oxygen availability is important to high-concentration methane oxidation.
- Microbial methane oxidation is affected by interactions of inorganic N and oxygen.
- Activity of Type I methanotrophs dominates under high methane concentration.

Abstract

Livestock slurry is a major source of atmospheric CH_4, but surface crusts harboring methane oxidizing bacteria (MOB) could mediate against CH_4 emissions. This study examined conditions for methane oxidation by *in-situ* measurements of O_2 and N_2O, as a proxy for inorganic N transformations, in intact crusts using microsensors. This was combined with laboratory incubations of crust material to investigate effects of O_2, CH_4, and inorganic N on methane oxidation, using $^{13}CH_4$ to trace C incorporation into lipids of MOB. Oxygen penetration into the crust was 2–14 mm, confining the potential for aerobic methane oxidation to a shallow layer. Nitrous oxide accumulated within or below the zone of O_2 depletion. With 10^2 ppmv CH_4 there was no O_2 limitation on methane oxidation at O_2 concentrations as low as 2%, whereas methane oxidation at 10^4 ppmv CH_4 was reduced at ≤ 5% O_2. As hypothesized, methane oxidation was in general inhibited by inorganic N, especially NO_2^-, and there was an interaction between N inhibition and O_2 limitation at 10^2 ppmv CH_4, as indicated by consistently stronger inhibition of methane oxidation by NH_4^+ and NO_3^- at 3% compared to 20% O_2. Recovery of ^{13}C in phospholipid fatty acids suggested that both Type I and Type II MOB were active, with Type I dominating high-concentration methane oxidation. Given the structural heterogeneity of crusts, methane oxidation activity likely varies spatially as constrained by the combined effects of CH_4, O_2, and inorganic N availability in microsites.

Introduction

In regions with intensive livestock production such as Western Europe and North America, up to 40% of livestock CH_4 emissions may be related to manure management (Francesco et al., 2013). In most cases, CH_4 capture and/or biofiltration is neither technically feasible nor economical (Melse and van der Werf, 2005), and more cost-effective alternatives must be considered. When manure is stored as liquid slurry, a dense floating crust is often formed either naturally from dry matter in the slurry, or by facilitation of admixing with chopped straw (Hansen et al., 2009). Studies have demonstrated a potential for aerobic methane oxidation in such surface crusts (Petersen and Ambus, 2006; Petersen et al., 2005), where diverse communities of methane-oxidizing bacteria (MOB) were also documented (Duan et al., 2014). These findings

suggest that surface crusts could act as a low-cost filter for manure-derived CH_4, but the physical, chemical, and biological regulation of methane oxidation inside crusts is largely unknown.

Methane oxidation potential depends on CH_4 and O_2 availability, which are highly variable due to the heterogeneous structure of the crust. Since surface crusts overlie liquid manure with a high methanogenic potential, they are typically high-CH_4 environments with concentrations far above the atmospheric level. We have observed up to 200 ppmv CH_4 in the stagnant atmosphere immediately above the surface crust (Y.F. Duan, unpublished data), and headspace CH_4 concentrations of 10^2 and 10^4 ppmv were used previously to simulate this range of CH_4 availability for MOB in laboratory incubations (Duan et al., 2013). Due to the often loose structure of the crust, sectioning for extraction and determination of *in situ* CH_4 availability by sampling are impractical. While a CH_4 biosensor has been described (Damgaard and Revsbech, 1997), it is not commercially available and has not been tested in heterogeneous environments such as surface crusts. In contrast to CH_4, the distribution of O_2 in surface crusts can be readily determined using a microsensor (Revsbech, 2005), as demonstrated by Hansen et al. (2009) and Nielsen et al. (2010) who investigated O_2 penetration into various slurry crusts.

Inhibition of methane oxidation by inorganic N is known from many environments (Bosse et al., 1993; Dunfield and Knowles, 1995; Wang and Ineson, 2003), as well as in surface crusts (Duan et al., 2013), but stimulation or no effect can also occur (Liu and Greaver, 2009). Livestock slurry and surface crusts are highly enriched in inorganic N (Table 1): the slurry phase may contain up to 200 mM ammoniacal N (NH_3/NH_4^+) (Nielsen et al., 2010; Sommer et al., 2007), while up to 35 mM NO_3^- (Hansen et al., 2009) and 98 mM NO_2^- (Nielsen et al., 2010) have been found at the oxic-anoxic interface in surface crusts. These concentration ranges (Table 1) represent a high variability of inorganic N at microsites within surface crusts due to fluctuations in water content as a result of precipitation and insolation (Nielsen et al., 2010). Due to this heterogeneity, bulk N concentrations are insufficient to characterize N distribution and thus potential interference with methane oxidation in microsites. While specialized sensors are available to determine micro-scale concentrations of NH_4^+, NO_2^-, and NO_3^- in aqueous environments (De Beer et al., 1997; De Beer

et al., 1991; Larsen et al., 1996), they are difficult to apply in unsaturated matrices such as surface crusts, where contact to liquid phase may be intermittent. Instead, the accumulation of N_2O in the crust was used in this study as a proxy for the distribution of N transformations and thus presence of inorganic N (Larsen et al., 1996), as both nitrification and denitrification can produce N_2O under sub-oxic conditions (Bollmann and Conrad, 1998).

The effects of environmental factors on methane oxidation in surface crusts will ultimately depend on the MOB present (Bodelier, 2011; Hu and Lu, 2015). Aerobic MOB have been conventionally categorized into Type I and Type II based on phylogenetic and functional traits, including the presence of signature 16-carbon (C_{16}) or 18-carbon (C_{18}) phospholipid fatty acids (PLFAs) (Hanson and Hanson, 1996). Type I MOB have been reported to thrive in N-sufficient, high-O_2 and low-CH_4 environments, whereas Type II MOB seem to favor the opposite (Amaral et al., 1995; Amaral and Knowles, 1995; Graham et al., 1993). It is still unclear to what extent CH_4, O_2, and inorganic N conditions will select one type of MOB over another in surface crusts, but previous studies found that Type I MOB are more abundant and diverse than Type II MOB in this environment (Duan et al., 2014; Hansen et al., 2009; Nielsen et al., 2013). In recent years, novel methane oxidation pathways, such as NO_2^- dependent anaerobic methane oxidation (Ettwig et al., 2010; Welte et al., 2016), as well as aerobic methane oxidation coupled with partial denitrification (Kits et al., 2015a; Kits et al., 2015b), have also been described, but the importance of these processes and the presence of relevant microorganisms in surface crusts remain unclear.

A main objective of this study was to examine the potential for microbial methane oxidation under realistic storage conditions by characterizing *in situ* distributions of O_2 and inorganic N transformations in a cattle slurry surface crust using microsensors. Effects and interactions of O_2 and inorganic N species with respect to methane oxidation could not be quantified *in situ* where microbial activities occur in micro-sites, and instead this was investigated under controlled laboratory conditions. Here, $^{13}CH_4$ was used as substrate, allowing ^{13}C stable isotope probing of PLFAs to study the involvement of Type I and Type II MOB in methane oxidation in surface crusts. Based on previous results (Duan et al., 2014; Duan et al., 2013) we hypothesized that

methane oxidation activity in surface crusts would be determined by both CH_4 and O_2 availability, and inhibited by inorganic N, and that Type I MOB would be primarily responsible for methane oxidation.

Materials and Methods

Microsensor Measurement of O_2 and N_2O Distribution

Dairy cattle slurry was collected from a full-scale manure storage facility in May, 2012, and transferred to two tanks at a pilot-scale storage system (Petersen et al., 2009) at Aarhus University (Foulum, Denmark). The slurry was stored for six weeks prior to the measurement in June, by which time a 5–6 cm thick surface crust with a stable structure had developed on top of the slurry. The development of surface crust reflected typical storage conditions, where a new crust is formed following the mixing of slurry in spring for field application.

Oxygen and N_2O concentration profiles in the surface crust were determined using, respectively, an O_2 and a N_2O microsensor with a tip diameter of 0.5 mm (both produced by Unisense, Aarhus, Denmark). Both microsensors were calibrated according to manufacturer's instructions. Detection limits for O_2 and N_2O were 0.3 µmol L^{-1} and 0.1 µmol L^{-1}, respectively.

A custom-made mounting system was used to place the microsensors over the surface crust (Supplemental Fig. S1). Oxygen profiles were recorded at 20-cm intervals from 20 to 180 cm along the 200-cm diameter of the storage tank. During measurement, the microsensor was introduced stepwise into the crust at 0.5 mm increments to a maximum depth of 30 mm using a motorized micromanipulator (Unisense). At each depth, the microsensor was stationary for 3 s to allow gas equilibration, and then the O_2 concentration was determined as the average of a 3 s reading. The signal was amplified by a multimeter (Unisense), and registered by the SensorTrace PRO v3.0 software (Unisense). Nitrous oxide profiles were determined at the same locations as O_2 profiles, but with a +2 mm offset to avoid any disturbance to the crust caused by the O_2 microsensor. The N_2O profiles were recorded with the same procedure as O_2 profiles, and initially over the

same depth. However, due to incidences of significant N_2O accumulation at 30 mm depth, the maximum depth was extended to 60 mm starting from the 5th profile (at 100 cm distance from the edge).

Due to the uneven surface of the crust, a fixed depth cannot accurately define the crust-air interface. For O_2, an abrupt decrease from atmospheric concentration defined the crust-air interface. For N_2O, which was produced inside the crust and diffused towards the atmosphere, the crust-air interface was defined as the depth where N_2O concentration dropped below the detection limit. Using these two criteria, the O_2 and N_2O concentration profiles were aligned.

Methane Oxidation in Response to O_2 and N Amendments

Surface crust was collected from a full-scale storage tank at the biogas plant of Aarhus University in March, 2012. The 10-cm surface crust had developed on slurry co-digested with maize silage. Homogenized crust samples were stored in closed plastic containers at 2°C until used for experiments within four weeks. Duan et al. (2013) showed that MOB can survive and recover activity under these storage conditions for at least three months.

Methane oxidation rates were determined by incubating 3-g crust samples in liquid media under a controlled atmosphere. To reduce background N, crusts were washed three times by vortexing with 20 mL deionized water followed by centrifugation at 10,000 × *g* for 10 min. The washing did not remove all NH_4^+, but the residual NH_4^+ was negligible compared to the received NH_4^+ amendment (Duan et al., 2013). Washed crust materials were transferred to 125 mL serum bottles, and resuspended in 20 mL basal salt (BS) medium prepared according to Whittenbury et al. (1970), but excluding N salts. The BS medium was dispensed using a customized system designed to remove dissolved O_2 and maintain anoxia during sample preparation (Supplemental Fig. S2).

Various treatments with combinations of different CH_4, O_2, and inorganic N were prepared (Table 2). For N amendments, pre-made solutions of $(NH_4)_2SO_4$, KNO_3, or KNO_2 were injected into the serum bottles to

achieve the desired N concentrations. The bottles were purged using a vacuum pump and refilled with helium, and this step was repeated three times to exhaust residual O_2. Then, air and ^{13}C-labeled CH_4 (99 atom% ^{13}C, ISOTEC, Miamisburg, OH, USA) were injected to achieve the desired headspace gas concentrations. To meet the requirement for CO_2 by some MOB, 4 mL pure CO_2 was also added (Acha et al., 2002). After gas injection, the bottles were mounted on a rotary shaker at 150 rpm for 30 min to allow for liquid-gas equilibration. Headspace O_2 concentrations were verified using an Agilent 3000A MicroGC (Hørsholm, Denmark) as described by (Petersen et al., 2009). For treatments with 0% O_2, residual headspace O_2 concentration was undetectable (< 10–20 ppmv). The bottles were then incubated on a rotary shaker at 200 rpm at ca. 21°C; Duan et al. (2013) had shown that there is no gas diffusion limitation under these incubation conditions. Headspace CH_4 concentrations were measured after 0, 2, 4, 6, 8, 24, 48, and 72 h using a Shimadzu 14B GC as described by Duan et al. (2013).

In each batch of assays, a control with crust material in N-free BS medium at atmospheric O_2 concentration was included to check for batch-to-batch variations in crust MOB activity, and a blank control without crust material to correct for loss of pressure during repeated gas samplings.

First-order rate constants for the first 8 h of incubation and relative activities were calculated according to Duan et al. (2013). First-order rate constants were compared between treatments using R v3.2.2 (R Core Team, 2015). For each CH_4 concentration, effects of O_2 and inorganic N, and their interaction, were analyzed by a two-way ANOVA. Differences between treatments were determined by Duncan's post-hoc multiple comparison test.

PLFA Extraction and GC-c-IRMS Analysis

After incubation with ^{13}C-labeled CH_4, selected crust samples were processed for PLFA analysis (Table 2). Prior to lipid extraction it was necessary to reduce the organic load since otherwise the humic material would bind the chloroform phase and prevent isolation of lipid-soluble compounds. Crust samples were vortexed and centrifuged for 10 min at 3000 × *g* to extract microbial cells. The supernatant was filtered

through 0.2 µm chloroform-soluble polycarbonate filters, and the material retained on the filter was used for lipid extraction. Hence, lipid results refer to the fraction of extractable low particle size material only. Polar lipid fatty acid methyl esters (FAMEs) from each filter were prepared as previously described by Petersen et al. (2002).

FAMEs were analyzed using a HP6890 GC (Agilent, Santa Clara, CA, USA) coupled via a GC combustion interface (Thermo Scientific, Bremen, Germany) in continuous flow mode to a Finnigan Delta^{PLUS} isotope ratio mass spectrometer (Thermo Scientific, Bremen, Germany). The oxidation reactor on the interface was maintained at 940 °C, the reduction reactor at 650 °C. Samples (1 µL) were injected at 240 °C in splitless mode. The column temperature was held at 50 °C for 2 min, then increased at 15 °C min^{-1} to 100 °C, subsequently at 2 °C min^{-1} to 220 °C, and finally at 15 °C min^{-1} to 240 °C, where the final temperature was held for 5 min. Separated compounds were measured against a CO_2 reference gas calibrated with reference to Vienna PeeDee belemnite. PLFAs were identified by relative retention time comparing samples against a FAME standard mix (Supelco 37 component FAME mix, 47885-U, Sigma Aldrich). All $\delta^{13}C$ values were corrected for the methanol C added during methanolysis:

$$\delta^{13}C_{FAME} = \frac{(N_{FA} + 1)\delta^{13}C_{FAME} - \delta^{13}C_{MeOH}}{N_{FA}}$$

where N_{FA} refers to the number of carbon atoms of the fatty acid component, $\delta^{13}C_{FAME}$ is the observed $\delta^{13}C$ value of the FAME, and $\delta^{13}C_{MeOH}$ is the $\delta^{13}C$ value of the methanol used for methanolysis (–37.7 ‰ ± 3.2 ‰). The $\delta^{13}C$ isotope ratios were converted to atom%, and atom% excess was then calculated by subtraction of an unlabeled control. The incorporation of $^{13}CH_4$ into membrane PLFAs (n_{13C}, nmol) was calculated as n_{13C} = (PA_{FAME} / PA_{ISME}) × n_{ISME} × (atom% ^{13}C excess), where PA_{FAME} and PA_{ISME} are peak areas of the FAME and internal standard *Me*19:0, respectively, and n_{ISME} (nmol C) is the concentration of the internal standard fatty acid. The lower limit of identified peaks corresponded to 0.1 ng ^{13}C g^{-1} crust material.

In view of the uncertain recovery of microbial cells following centrifugation and filtration, statistical testing of treatment effects was not performed, and results will only be presented as means ± standard errors.

Results

Oxygen and N_2O Distribution in Natural Surface Crusts

Surface crusts from the two storage tanks showed a high spatial variability in shape and penetration depth of individual O_2 and N_2O profiles, yet the distribution patterns were qualitatively similar between the two tanks. Thus, results presented here are from one of the storage tanks only (Fig. 1).

Oxygen penetration depth varied from 2 to 14 mm, with either a steep or more gradual decline in concentration. Irregularities such as a secondary increase following the initial decline were also observed (e.g., at 60 cm).

Nitrous oxide profiles showed peak concentrations at 5–25 mm depth below the crust-air interface. Some N_2O profiles consisted of more than one zone of N_2O accumulation (e.g., at 60, 100, and 160 cm). Maximum N_2O accumulation often coincided with sub-oxic or anoxic zones indicated by O_2 profiles. In a few cases (e.g., at 60 and 180 cm), N_2O production took place where O_2 availability was relatively high.

Response of CH_4 Oxidation to O_2 Concentrations

Figure 2 shows the changes in headspace CH_4 concentrations during the 72-h incubation study with different initial O_2 levels and two initial CH_4 levels. For both 10^2 and 10^4 ppmv CH_4, consistent methane consumption throughout the incubation was observed only at 20% initial O_2, with a > 90% decline in headspace CH_4 concentrations after 72 h. In treatments with ≤ 5% initial O_2, CH_4 consumption was generally observed within the first 24 h, followed by net CH_4 accumulation. At 0% initial O_2, net CH_4 production was observed throughout the incubation.

189 The strength of methane oxidation activity was expressed as first-order rate constants during the first 8 h of
190 incubation (Fig. 3). At 10^2 ppmv initial CH_4 there were no significant differences in first-order rate constants
191 at O_2 levels from 20% down to 2%, but at 1% O_2 the rate was significantly impaired. At 10^4 ppmv initial CH_4,
192 methane oxidation rates were significantly reduced at lower O_2 levels, though not significantly different
193 between 2% and 1% O_2.

194 ## *Response of CH_4 Oxidation to Interactions between O_2 and Inorganic N*

195 When samples with manipulated O_2 and CH_4 concentrations were amended with inorganic N, complex
196 patterns in microbial methane oxidation were observed. For each of the two initial CH_4 concentrations, two-
197 way ANOVA showed significant effects of O_2 concentrations and N amendments, as well as their interaction,
198 on methane oxidation rates.

199 At 10^2 ppmv CH_4, first-order rate constants in all N-amended samples were lower than in the N-free control
200 (Table 3). The inhibition was strengthened at increasing concentrations of both NH_4^+ and NO_3^-. Samples
201 treated with NO_3^- consistently showed less inhibition than treatments receiving other N salts, whereas NO_2^-
202 was a potent inhibitor as indicated by similar inhibitions with 1 mM NO_2^- and 50 mM NH_4^+. At 3% O_2,
203 inhibition by individual N species and concentrations was slightly stronger than at 20% O_2, but the difference
204 was not always statistically significant.

205 At 10^4 ppmv CH_4 the order of inhibition by different N species was similar to that at 10^2 ppmv CH_4, with NO_2^-
206 as the strongest inhibitor (Table 3). Yet, several NO_3^- treatments caused a weak stimulation rather than
207 inhibition as compared to the N-free control. Generally, the N amendments inhibited methane oxidation at
208 20% O_2, but not at 3% O_2, where low rates were already observed in the N-free control. One exception,
209 though, was the inhibition caused by the treatment with 50 mM NH_4^+.

^{13}C Incorporation into C_{16} and C_{18} PLFAs

The yields of PLFA varied considerably between samples, and ^{13}C incorporation was below the detection limit for several PLFAs, precluding a detailed quantitative analysis. Instead of absolute PLFA concentrations, an index based on peak area was calculated (Fig. 4, a1–a4). Recovery of ^{13}C PLFAs was consistently low in incubations with 10^2 ppmv CH_4 (Fig. 4, a1). At 10^4 ppmv CH_4 there was an 8–9 times higher ^{13}C recovery in PLFAs at 20% and 3% O_2 than at 1% O_2 (Fig. 4, a2). While NH_4^+ and NO_3^- considerably reduced ^{13}C recovery, NO_2^- caused no or only moderate inhibition of ^{13}C recovery (Fig. 4, a3 and a4). The total recovery of ^{13}C was 5–15 times higher with NO_2^- amendment than with the other two N species.

Two ^{13}C-labeled PLFA clusters, C_{16} and C_{18}, were defined in accordance with the predominance of these PLFAs in Type I and Type II MOB, respectively (Bodelier et al., 2009). The C_{16} cluster included peaks identified as 16:0, 16:1ω6, 16:1ω7, and 16:1ω8, whereas the C_{18} cluster included 18:0, 18:1ω7, 18:1ω9, and probably also small peaks of 18:1ω8. Also, 16:1ω6 probably co-eluted with 10*Me*16:0, and 16:1ω8 with i17:0, but this did not influence the calculated ^{13}C incorporation for the cluster.

Due to the low recovery of total ^{13}C PLFA (Fig. 4, a1) and low percentage (3–9%) of ^{13}C in C_{16} and C_{18} clusters at 10^2 ppmv CH_4 (Fig. 4, b1), no detailed interpretation of these results was possible. At 10^4 ppmv CH_4 the recovery of ^{13}C in the C_{16} and C_{18} clusters together accounted for an average of 52% of ^{13}C recovered in PLFAs. A higher percentage of ^{13}C was always recovered in the C_{16} than in the C_{18} cluster, and the percentage of the C_{16} cluster was particularly high in treatments with NO_2^- amendment (Fig. 4, b3 and b4).

Discussion

Due to the heterogenous nature of surface crusts, microbial activities within this environment are controlled by physical and chemical properties of individual microsites rather than overall bulk properties. However, detailed analysis of surface crusts is challenged by the often loose and fibrous structure of the material, and this was also the case with the straw-containing cattle slurry crust used in this study. Therefore, we chose to

characterize O_2 and inorganic N distributions *in situ* by microsensors, while regulation of microbial activities were investigated by controlled laboratory incubations. For logistic reasons, the *in situ* gas measurements and laboratory incubations were performed using different surface crusts. However, previous studies have shown that crusts of different origin are qualitatively similar with respect to, e.g., depth of O_2 penetration and the presence of MOB (Duan et al., 2014; Hansen et al., 2009; Nielsen et al., 2010; Nielsen et al., 2013), and results from the two parts are therefore analyzed and discussed together.

Effects of O_2 Limitation and N Amendments

Methane oxidation kinetics are complex as the reaction involves two substrates, CH_4 and O_2 (Cai and Yan, 1999). We were not able to monitor O_2 concentrations during incubation, but instead calculated O_2 consumption by MOB based on the amounts of CH_4 consumed, the stoichiometry of methane oxidation (Urmann et al., 2007), and the diffusion coefficients of CH_4 and O_2 in water (Broecker and Peng, 1974) (Supplemental Table S1). These calculations suggested that there was no diffusional limitation of O_2 for methane oxidation, and that the amounts of O_2 used for methane oxidation were < 10% of the available O_2 even at 1% initial O_2. Thus, depletion of O_2 during incubation must have been mainly due to aerobic processes other than methane oxidation, i.e., any O_2 limitation for MOB activity reflected competition for O_2 against other aerobes. Headspace CH_4 concentrations decreased exponentially during the first 8 h, indicating that the rates of CH_4 uptake depended mainly on CH_4 availability. Therefore, the reaction was approximated by first-order reaction kinetics, which have also been used previously in studies of microbial methane oxidation (De Visscher et al., 1999; King and Schnell, 1994; King and Schnell, 1998; Petersen and Ambus, 2006). The apparent first-order kinetics suggest that the rate of CH_4 uptake, and thus demand for O_2, was proportional to the CH_4 concentration, which explains our observation that methane oxidation became more affected by O_2 limitation at 10^4 ppmv than at 10^2 ppmv CH_4 (Fig. 3).

The neutral to alkaline pH of slurry (Nielsen et al., 2010; Petersen and Ambus, 2006) suggests the presence of free ammonia (NH_3), which is a competitive inhibitor for methane oxidation (Carlsen et al., 1991; Gulledge

and Schimel, 1998). In both the present (Table 3) and a previous study (Duan et al., 2013), inhibition by ammonia was less at 10^4 compared to 10^2 ppmv CH_4, suggesting that competitive inhibition is important for effects of ammonia. The observations that NO_2^- was a more potent inhibitor of methane oxidation than NO_3^- was also consistent with the report by Duan et al. (2013), where the concentration of NO_3^- resulting in 50% inhibition was 100-fold higher than that of NO_2^-. It is likely that these N species both inhibit methane oxidation *via* nitrite toxicity (Stein and Klotz, 2011).

We further tested interactions of N inhibition with O_2 availability by comparing relative activities. If there were an interaction between O_2 and inorganic N, a given N amendment would result in different degree of inhibition at 20% and 3% O_2 concentrations. At 10^2 ppmv initial CH_4, 10 mM, but not 50 mM, NH_4^+ or NO_3^- amendments caused a stronger inhibition at 3% than at 20% O_2 (Table 3), confirming an interaction between N inhibition and O_2 limitation at low N concentrations. At 10^4 ppmv CH_4, the fact that N amendments generally inhibited methane oxidation at 20% O_2 but not further at 3% O_2 suggested that high-concentration methane oxidation is more sensitive to O_2 limitation rather than to N inhibition.

Generally, as an essential substrate for aerobic methane oxidation, O_2 has a direct and immediate effect on aerobic methanotrophic activity. On the other hand, the mechanism of N inhibition on methane oxidation is much more complex and may include immediate toxicity to cell growth and enzyme synthesis, as well as delayed influence on microbial community composition (Bodelier and Laanbroek, 2004). The mechanisms behind interactions between controlling factors in surface crusts could not be explained with the data presented here, and more research is needed to further elucidate this matter.

^{13}C PLFA Signatures for MOB

Type I and Type II MOB produce unique membrane PLFAs, 16:1ω8 and 18:1ω8, respectively (Bodelier et al., 2009; Hanson and Hanson, 1996). These signature PLFAs are not always present, or present only in low amounts. However, there is also a general predominance of C_{16} and C_{18} PLFAs among Type I and Type II MOB, respectively (Bodelier et al., 2009), and this has been used to evaluate sources of methane oxidation

by stable isotope probing (Qiu et al., 2008). In the present study, both types of MOB were active, with Type I MOB dominating the methanotrophic activity especially where CH_4 availability was high (Fig. 4, b1 and b2). Molecular analyses of microflora in other crust materials likewise suggested that Type I MOB dominated the methanotrophic community in terms of both diversity and abundance (Duan et al., 2014).

Interestingly, the incorporation of ^{13}C into PLFAs was high in surface crusts incubated with NO_2^- as compared to those with other N species (Fig. 4, a3 and a4), especially considering that crusts incubated with NO_3^- had over two-fold higher $^{13}CH_4$ uptake than NO_2^- treatments (data not shown). Roslev et al. (1997) reported that NH_4^+ decreased C conversion efficiency and increased respiration of C assimilated by MOB, which is consistent with the low ^{13}C recovery from NH_4^+ treatments in this study. However, the mechanism by which NO_3^- and NO_2^- could interfere with C assimilation for MOB remains unclear. Alternative pathways for CH_4 uptake may have contributed to the particularly high ^{13}C assimilation in NO_2^- amended crusts. The microorganism *Candidatus Methylomirabilis oxyfera* is able to couple anaerobic methane oxidation with nitrite reduction (Ettwig et al., 2010), and may be widespread in natural environments (Ettwig et al., 2009; Wang et al., 2012; Zhu et al., 2012). We recovered 10*Me*$C_{16:0}$ in the present study, which is characteristic of the lipid profile of *M. oxyfera* (Kool et al., 2012). Still, more concrete evidence, such as the recovery of specific gene markers of *M. oxyfera* (Luesken et al., 2011), is needed to confirm the presence of *M. oxyfera* in surface crusts. More recently, gammaproteobacterial (Type I) methanotrophs *Methylomonas denitrificans* and *Methylomicrobium album* have been reported to be able to oxidize methane under hypoxia using oxidized nitrogen as electron acceptor (Kits et al., 2015a; Kits et al., 2015b). The involvement of such a process would be consistent with the high proportion of C_{16} PLFAs recovered from NO_3^- and NO_2^- treatments, and the genera *Methylomonas* and *Methylomicrobium* are widespread (Knief, 2015) and were indeed present in surface crusts (Duan et al., 2014). However, there was no direct evidence in the present study to evaluate the presence of these specific strains.

In-situ O_2 and N_2O Distribution and Implications for CH_4 Oxidation

Oxygen distribution in surface crusts vary over short distances, as shown in this and other studies (Hansen et al., 2009; Nielsen et al., 2010). Despite this variation, the depth of O_2 penetration is generally shallow and was never more than 25% of the thickness of the crust (Nielsen et al., 2010). The restriction of significant O_2 penetration is likely due to surface crusts being a floating organic structure on top of liquid slurry, where the bottom of the crust is always saturated, while aerobic processes actively consume O_2 in upper layers. Nielsen et al. (2010) proposed that trapping of gases formed in the slurry could elevate the crust above the liquid slurry phase and, as a result, improve O_2 penetration. Measurements of O_2 at a fixed depth over 48 h did indicate gas pockets that lifted the crust, but they were intermittently deflated (Supplemental Fig. S3). Structural voids in the crusts, such as pores and crevices, could also provide access for O_2 to deeper parts of the crusts, and weather conditions such as precipitation and drought will influence O_2 permeability by altering the wetness of the crust (Hansen et al., 2009).

The present study observed N_2O levels as high as 100 µmol L^{-1}, which was far above the N_2O concentrations commonly found in other environments. For example, Baral et al. (2014) and Zhou et al. (2016) reported N_2O concentrations of < 5 µmol L^{-1} near the soil surface. Careful examination of potential interferences to the microsensor is therefore warranted. Surface crusts may contain up to 300 µmol L^{-1} of H_2S (Nielsen et al., 2010), and H_2S is known to affect the signal of N_2O microsensors. However, the microsensor used here is equipped with an alkaline oxygen guard which converts incoming H_2S to ionic forms, and this offers some protection to the cathode from H_2S (Andersen et al., 2001). With the same type of microsensor used in this study, Andersen et al. (2001) found a reduction in the sensitivity towards N_2O with increasing concentrations of H_2S up to 350 µmol L^{-1}, indicating that any H_2S interference would result in lower, not higher, N_2O readings. Therefore, the high N_2O concentrations observed were not likely to be a result of H_2S interference. In the above mentioned studies where soil N_2O concentrations were <5 µmol L^{-1}, the corresponding N_2O emissions were 3.6 and 25 µmol m^{-2} h^{-1} (Baral et al., 2014; Zhou et al., 2016). For livestock slurry with surface crusts, N_2O emissions as high as 393–1,429 µmol m^{-2} h^{-1} have been reported (Hansen et al., 2009;

329 Sommer et al., 2000). Considering the relationship between N_2O concentration and emission, N_2O
330 concentrations up to 100 μmol L^{-1} in surface crusts seem plausible.

331 Maximum N_2O accumulation occurred near the oxic-anoxic interface in most cases, and therefore both
332 nitrification and denitrification were potential sources of N_2O (Braker and Conrad, 2011). Law et al. (2012)
333 found a correlation between N_2O production and ammonia oxidation rate, possibly as a result of nitrifier
334 denitrification to conserve O_2 or prevent NO_2^- toxicity (Lawton et al., 2013), and similar mechanisms could
335 lead to N_2O accumulation via ammonia oxidation in surface crusts. At or below oxic-anoxic interfaces,
336 incomplete heterotrophic denitrification was likely the main source of N_2O due to intolerance of N_2O
337 reductase towards trace O_2 (Thomson et al., 2012). Nitrous oxide could also be released as a terminal
338 product from methane oxidation coupled with partial denitrification by *M. denitrificans* (Kits et al., 2015b)
339 and *M. album* (Kits et al., 2015a; Nyerges et al., 2010), which can be enabled by hypoxia developed at the
340 oxic-anoxic interfaces. Denitrification could also act as a sink for NO_3^- or, particularly, NO_2^- which is a strong
341 inhibitor of aerobic methane oxidation. The ubiquitous presence of N_2O in the surface crusts indicated active
342 transformations of N species which could interfere with methane oxidation. Yet, as seen from the incubation
343 experiments, the effect of N species on MOB may be either inhibitory or stimulatory depending on other
344 factors. Moreover, Type I MOB utilizing NO_2^- and/or NO_3^- for methane oxidation are presumably more
345 resilient to N inhibition than others (Zhu et al., 2016). In support of this, *M. denitrificans* and *M. album* have
346 been shown to tolerate and grow under 10 mM NO_3^- (Kits et al., 2015b) and 2.5 mM NO_2^- (Nyerges et al.,
347 2010), respectively. Also, Hu and Lu (2015) found that, while NH_4^+ and NO_3^- both stimulated Type I MOB as
348 determined from *pmo*A gene copy numbers, Type II MOB were inhibited by NH_4^+ as concentrations
349 increased.

350 Clearly, various physio-chemical and biological properties are involved in regulating methane oxidation in
351 surface crust. Firstly, the heterogeneous structure of the crust adds complexity to the distribution of gases.
352 Secondly, there are complex interactions between CH_4 and O_2 levels, and inorganic N species and
353 concentration, with respect to methanotrophic activity. In parts of the crust where CH_4 availability is

relatively low, moderate O_2 limitation probably has little impact on aerobic methane oxidation activity, whereas N inhibition could be significant depending on N species and concentration. In contrast, in parts of the crust with high CH_4 availability, O_2 limitation is likely the main control of aerobic methane oxidation, and inhibition due to inorganic N is only important where O_2 is not limiting.

Conclusions

Microsensor measurements of *in situ* O_2 and N_2O profiles revealed shallow penetration of O_2 into slurry surface crusts and active N transformations around oxic-anoxic interfaces. Laboratory incubations suggested that O_2 availability was more important to high-concentration than low-concentration methane oxidation, and there were complex interactions between inorganic N and O_2 limitation. The incorporation of ^{13}C from CH_4 into membrane PLFAs indicated that both Type I and Type II MOB were actively involved in methane oxidation, but with Type I MOB dominating the activity at high CH_4 concentrations. These observations together imply that manipulation of storage conditions to increase headspace CH_4 concentration, as proposed by Petersen and Miller (2006), could stimulate methane oxidation by Type I MOB in the upper parts of the crust where O_2 is non-limiting and mineral N availability low.

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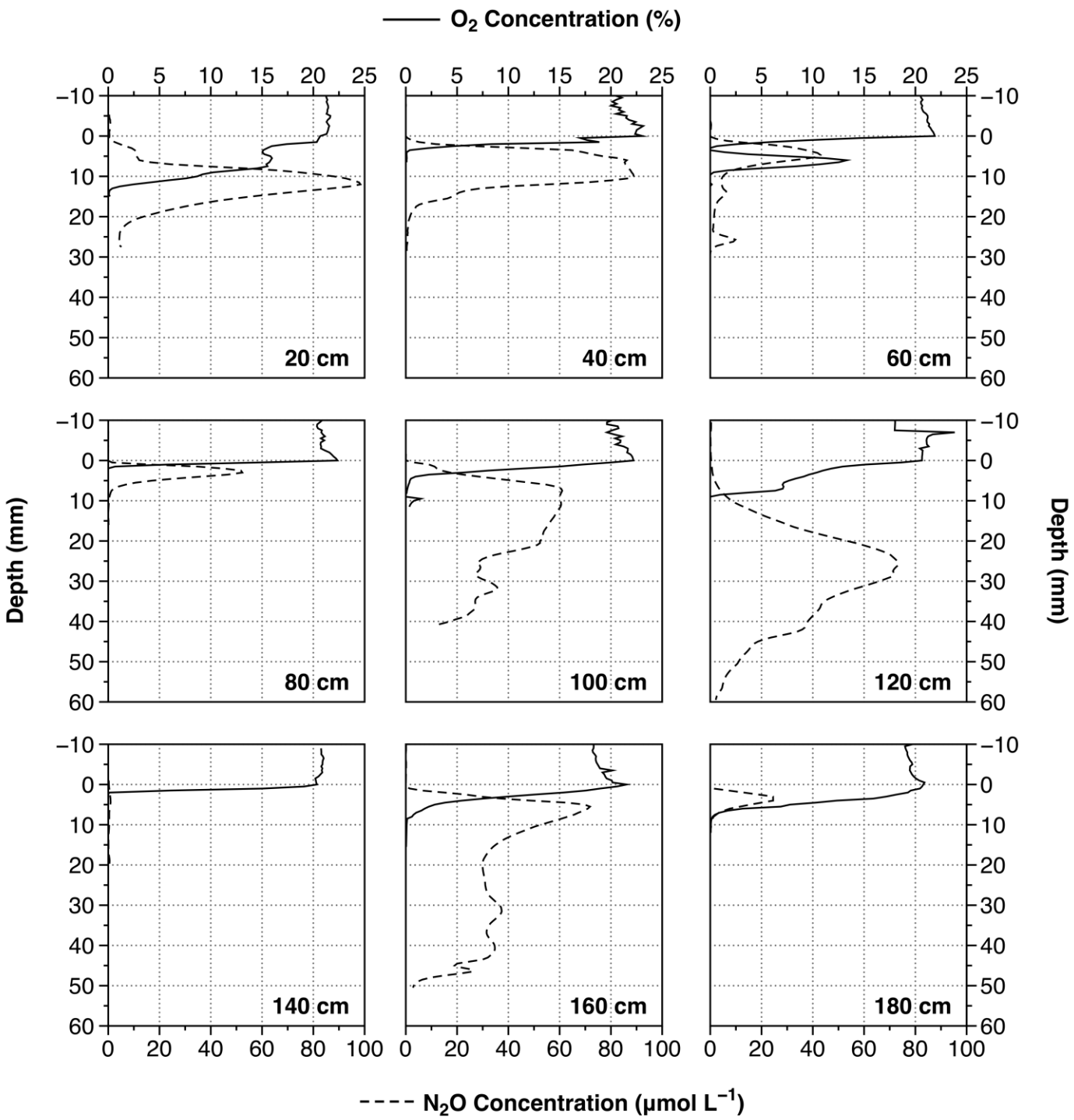

Figure 1: Profiles of oxygen (O_2, solid line, top X-axis) and nitrous oxide (N_2O, dashed line, bottom X-axis) profiles in a surface crust measured using microsensors at 20 cm intervals along the 200-cm diameter of a slurry storage tank. Distances of the sampling points from the edge of the tank are indicated on the bottom-right corner of each panel. Oxygen was traced to a depth of 30 mm in all cases; N_2O was traced to a depth of 30 mm at 20–80 cm and to a depth of 60 mm at 100–180 cm.

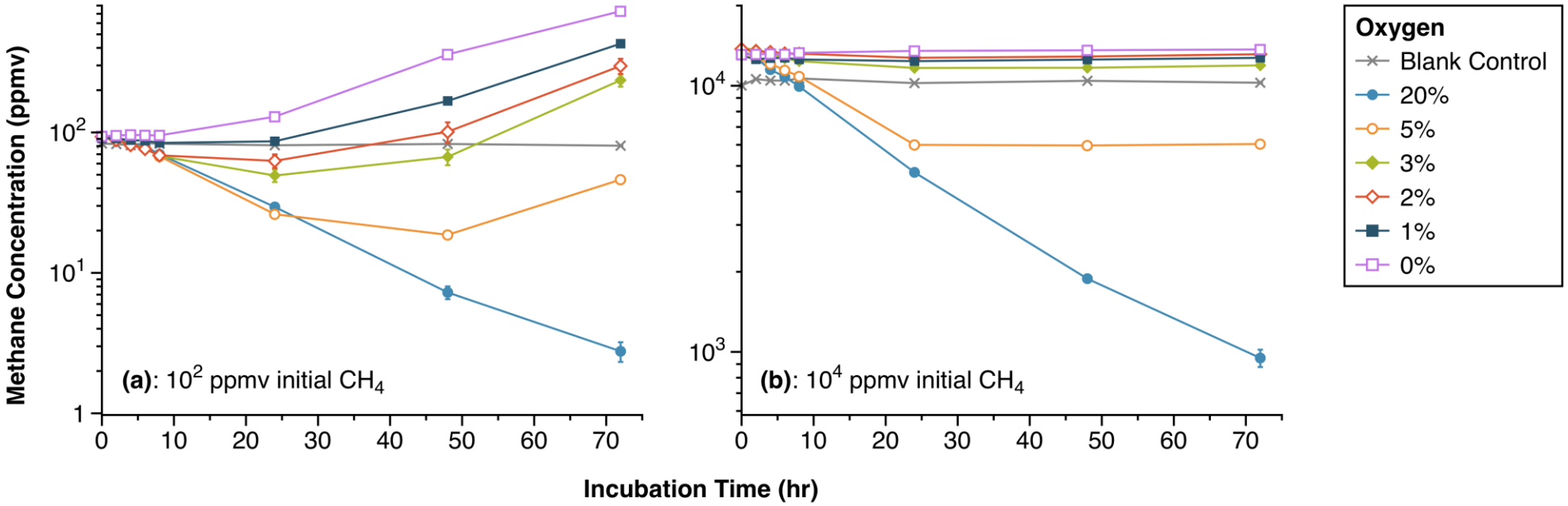

Figure 2: Dynamics of CH_4 concentrations during a 72-h incubation of slurry surface crusts with initial O_2 concentrations of 20, 5, 3, 2, 1, and 0%, and with initial CH_4 concentrations of 10^2 ppmv (a) and 10^4 ppmv (b). Blank control contained 20% initial headspace O_2 and no crust material. Each point represents the mean of triplicate assays, and error bars show standard error.

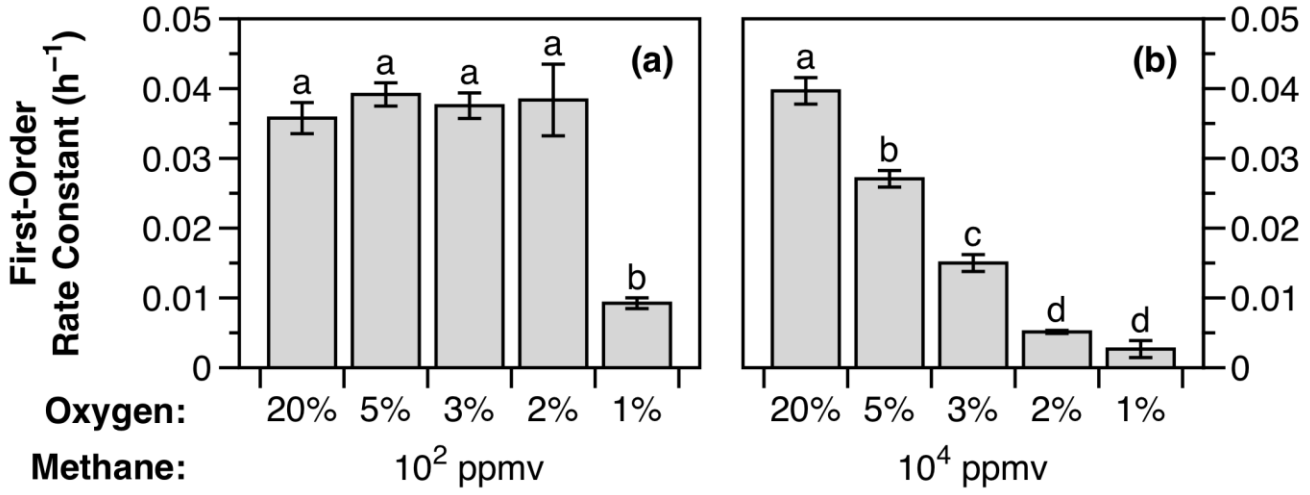

Figure 3: First-order rate constants of potential CH_4 oxidation under different O_2 and CH_4 concentrations during the first 8 h of incubation. The bars show the mean of triplicate assays, and the error bars show standard error. For each CH_4 concentration, bars denoted with the same letter on top are not significantly different at $\alpha = 0.05$.

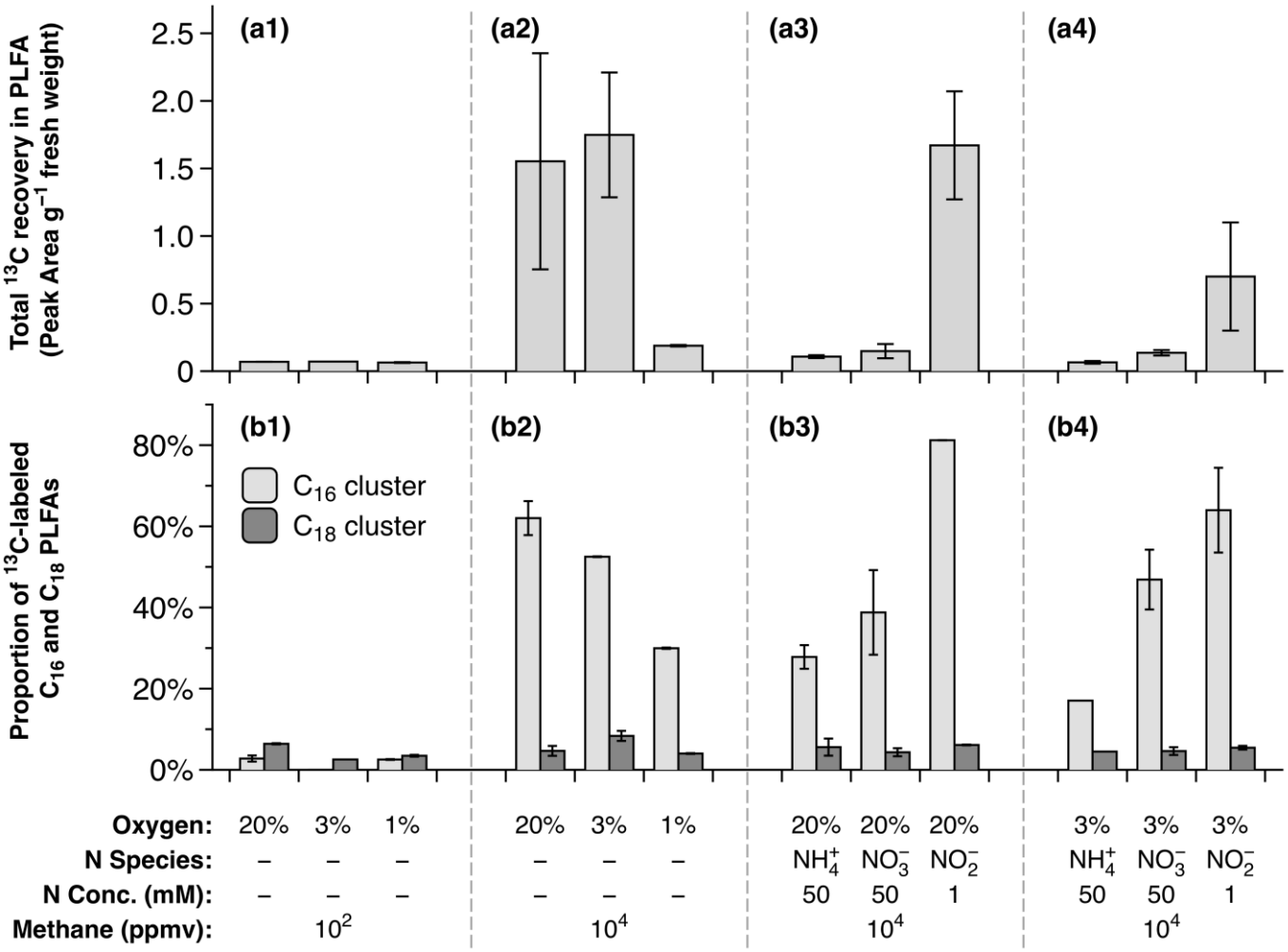

Figure 4: Total recovery of ^{13}C in PLFA (expressed as peak area per gram fresh crust) derived from incubation of surface crusts with $^{13}CH_4$ (a1–a4), and proportions of ^{13}C incorporation into PLFA belonging to C_{16} or C_{18} clusters (b1–b4) at different combinations of O_2, CH_4, and inorganic N availability. Data shown are means and standard errors of two replicates.

Table 1: Selected chemical properties of various livestock slurries and surface crusts from previous studies.

Sample	Surface Crust			Slurry	
	NH_4^+ µmol kg^{-1} WW*	NO_3^- µmol kg^{-1} WW	NO_2^- µmol kg^{-1} WW	pH	NH_4^+ mM
Cattle slurry surface crusts developed for 3 years (Duan et al., 2013)	116.3	370.9	n.a.#	n.a.	n.a.
Various cattle and swine slurries and surface crusts (Nielsen et al., 2010)	n.a.	7– 3,602	2–98,000	6.96–7.7	84–205
Surface crusts of various dryness (Hansen et al., 2009)	n.a.	290–35,000	80–1,990	7.7	175
Various cattle and swine slurries (Sommer et al., 2007)	n.a.	n.a.	n.a.	n.a.	79–257
Surface crusts (Petersen et al., 2006)	1,432–54,923	91–16,378	3–50	7.13–8.89	1.7–69
Digested cattle slurry (Clemens et al., 2006)	n.a.	n.a.	n.a.	7.4–7.8	85–127

* WW, wet weight.

n.a., value not reported.

Table 2: Combinations of CH_4, O_2, and inorganic N amendments used in this study. The values indicate the number of replicates prepared. Treatments marked with asterisks (*) were used for analysis of ^{13}C-labelled phosphate lipid fatty acids (PLFAs).

N Species and Concentrations		CH_4:	10^2 ppmv						10^4 ppmv					
		O_2:	0%	1%	2%	3%	5%	20%	0%	1%	2%	3%	5%	20%
None			3	3*	3	3*	3	3*	3	3*	3	3*	3	3*
NH_4^+	10 mM					2		2				2		2
	50 mM					2		2				2*		2*
NO_3^-	10 mM					2		2				2		2
	50 mM					2		2				2*		2*
NO_2^-	1 mM					2		2				2*		2*

Table 3: First-order rate constants (h^{-1}) of CH_4 oxidation in slurry surface crust samples under different O_2 concentrations and N amendments. Numbers in parentheses indicate relative activity (the ratio of the treatment activity as compared to the activity of the N-free control). Under each CH_4 concentration, values followed by the same letter are not significantly different at $\alpha = 0.05$.

N Species and Concentrations		First-Order Rate Constants (h^{-1})			
		10^2 ppmv CH_4		10^4 ppmv CH_4	
		20% O_2	3% O_2	20% O_2	3% O_2
None		0.036 (1.00) a	0.037 (1.00) a	0.040 (1.00) b	0.016 (1.00) e
NH_4^+	10 mM	0.015 (0.42) d	0.010 (0.27) ef	0.031 (0.78) c	0.017 (1.06) e
	50 mM	0.007 (0.19) fg	0.005 (0.14) g	0.013 (0.33) e	0.005 (0.31) f
NO_3^-	10 mM	0.029 (0.81) b	0.022 (0.59) c	0.054 (1.35) a	0.022 (1.38) d
	50 mM	0.017 (0.47) d	0.013 (0.35) de	0.028 (0.70) c	0.021 (1.31) d
NO_2^-	1 mM	0.008 (0.22) fg	0.008 (0.22) fg	0.016 (0.40) e	0.017 (1.06) e

Supplemental Material

Methanotrophic activity in slurry surface crusts as influenced by CH_4, O_2, and inorganic N

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(a). The 200-cm long aluminum bar with distance marks.

(b). The aluminum bar installed on the inner rim of the slurry tank.

(c). The motorized micromanipulator mounted on the aluminum bar.

(d). The microsensor fixed to the micromanipulator.

Figure S1: Custom-made mounting system for *in situ* measurement of O_2 and N_2O profiles using microsensors.

A 200-cm long aluminum bar with mm-scale distance marks (a) was installed on the inner rim of the storage tank, approximately 40 cm above the surface crust (b). A computer-controlled motorized micromanipulator (Unisense, Aarhus, Denmark) capable of vertical movement was mounted on a custom-made rack, which could be moved manually along the length of the aluminum bar (c). The microsensor was fixed to the micromanipulator using a rubber-lined clamp (d). When installed at the initial position for measurement, the tip of the microsensor was approximately 1 cm above the surface of the crust. During measurements, a cover was placed loosely over the storage tank to avoid heating of the surface crust by direct insolation.

When measurement at one sampling point was completed, the microsensor was retreated to initial position and temporarily detached from the micromanipulator to protect the tip from breaking during movement, and reinstalled after the micromanipulator had been moved to the next sampling point.

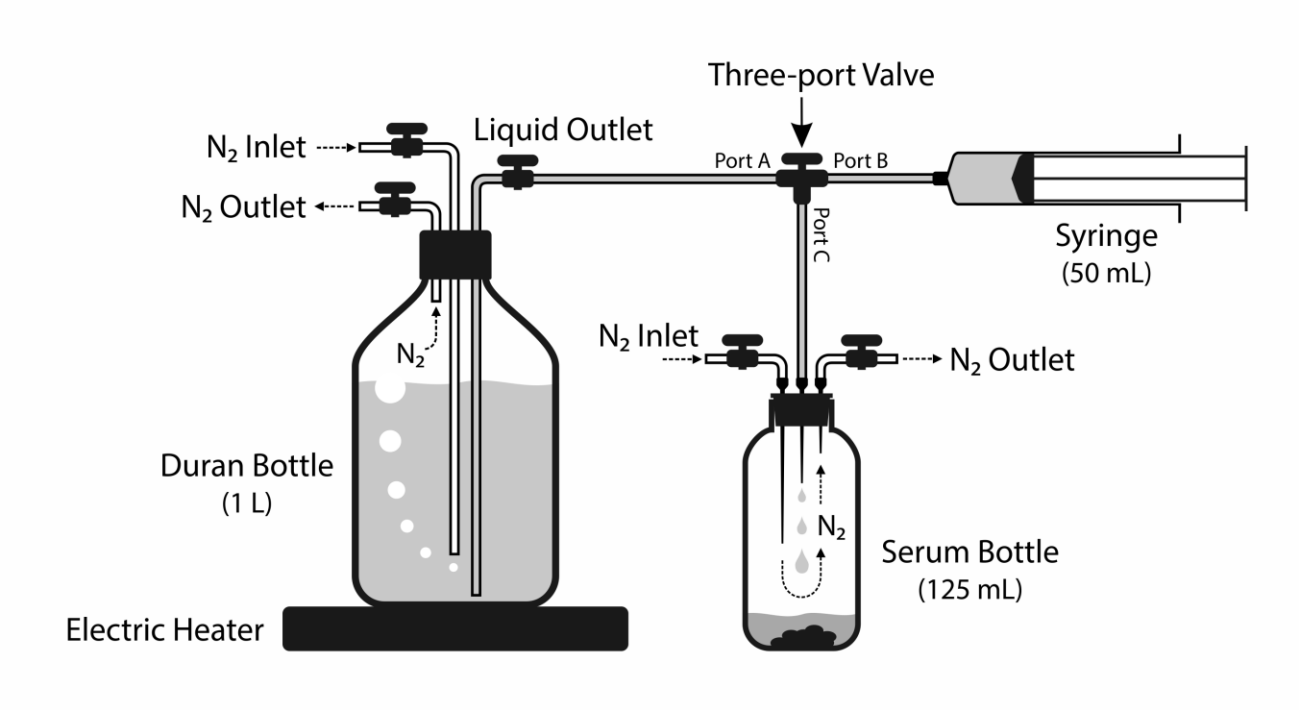

Figure S2: Diagram of the setup to prepare and distribute anoxic incubation medium.

A Duran bottle was modified to have three plastic tubes inserted through the cap: one as N_2 inlet, one as N_2 outlet, and the third as liquid outlet. The Duran bottle was filled with BS medium to ca. 70% of its volume and placed on an electric heater with the cap tightly secured. The BS medium was heated to the boiling point while bubbled with pure N_2 for at least 30 min, and then cooled to room temperature still under N_2 bubbling. Then, the N_2 outlet was closed and the liquid outlet valve was opened, and the entire tubing was flushed and filled with anoxic BS medium by the build-up of gas pressure inside the Duran bottle. When distributing BS medium to the serum bottle, a needle connected to pure N_2 flow was first inserted into the serum bottle through the rubber stopper and then, a second needle was inserted as N_2 outlet. The serum bottle was flushed with N_2 for 10 sec, and then a third needle connected to the three-port valve was inserted. Ports A and B of the three-port valve were opened while C was closed, and the syringe was filled with 20 mL incubation medium. Then, ports B and C were opened and A was closed and the incubation medium was injected from the syringe into the serum bottle while the bottle was being flushed by pure N_2.

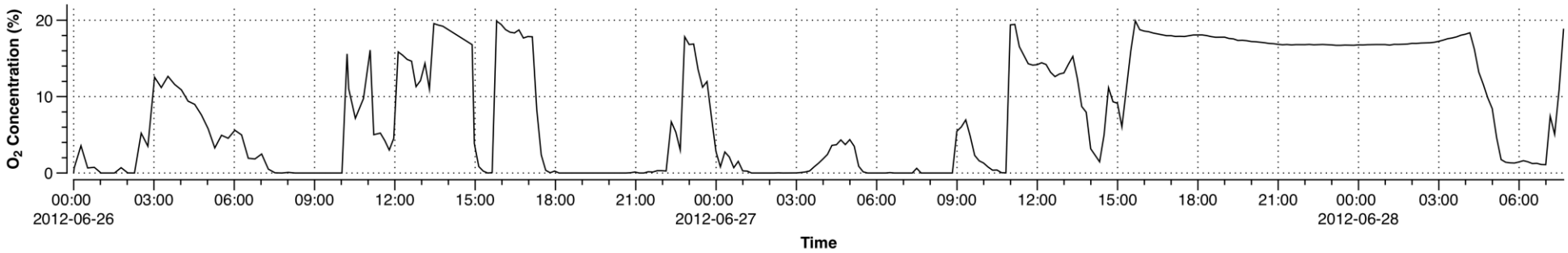

Figure S3: Dynamics of O_2 concentration at a fixed depth in a surface crust over 48 hours. The storage tank was covered to eliminate wind effect but was passively ventilated. The microsensor was initially fixed in a position where the tip just touched the surface of the crust. Oxygen concentrations were measured every 10–15 min as described in the manuscript. The peaks in O_2 profile showed that the microsensor was alternately exposed to atmospheric O_2 and more anaerobic conditions. This indicated that the surface of the crust periodically rose up and moved down, likely due to inflation and deflation of gas pockets underneath the crust.

Table S1: Oxygen Consumption during CH_4 oxidation at 10,000 ppmv initial CH_4.

Initial State (time 0)		**After 8 h**		
Headspace O_2 (%)	Dissolved O_2 (μM)	CH_4 Consumed (μM)	O_2 Consumed (μM)	O_2 Remaining (μM)
20	256.0	4.92	9.84	246.2
3	38.4	1.95	3.90	34.5
1	12.8	0.57	1.14	11.7

- Stoichiometry of CH_4 oxidation is:
  $$\mathrm{CH_4} + (2 - x)\mathrm{O_2} \longrightarrow (1 - x)\mathrm{CO_2} + x\mathrm{CH_2O} + (2 - x)\mathrm{H_2O}$$
  , where *x* is the fraction of carbon that is assimilated into biomass (CH_2O) (Urmann et al., 2007). Therefore, CH_4 to O_2 ratio in CH_4 oxidation theoretically ranges between 1:1 (100% C assimilation) and 1:2 (no C assimilation). In this calculation, we assumed maximum O_2 consumption, i.e. a CH_4 to O_2 ratio of 1:2.

- Henry's Law constant (*K*) for O_2 and CH_4 are 1.28 and 1.34 mmol L^{-1} atm^{-1}, respectively.

- Dissolved O_2 was calculated as: $K \times$ [Headspace O_2].

- Diffusivities of dissolved O_2 and CH_4 in water at 20°C are 2.06 and 1.75×10^{-5} cm^2 sec^{-1}, respectively (Broecker and Peng, 1974). Therefore, there's no diffusion limit of O_2 for CH_4 oxidation.